

# Plants, Mycorrhizal Fungi and Endobacteria: a Dialog Among Cells and Genomes

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*This review focuses on mycorrhizas, which are associations between fungi and the roots of 90% of terrestrial plants. These are the most common symbioses in the world; they involve about 6000 species of fungi distributed through all the fungal phyla and about 240,000 species of plants, including forest and crop plants. Thanks to mycorrhizal symbiosis and nutrient exchanges, regulated by complex molecular signals, the plant improves its vegetative growth, while the fungus accomplishes its life cycle. Molecular and cellular analyses demonstrate that during colonization the cellular organization of the two eukaryotes is completely remodeled. For example, in cortical cells, structural modifications involve both the host and the microbiont. Recent studies revealed that in arbuscular mycorrhizas (AM), system complexity is increased by the presence of a third symbiont: a bacterium living inside the fungus. The presence of this resident genome makes the investigation of the molecular dialogues among the symbiotic partners even more complex. Molecular analysis showed that the bacterium has genes involved in the acquisition of mineral nutrients. The experimental data support the current view that mycorrhizal symbioses are often tripartite associations.*

Endosymbioses are excellent systems with which to investigate the dialog among genomes and cells. The aim of this short report is to demonstrate that mycorrhizas are of particular interest in an evolutionary context, because they

offer a fine example of interactions between plants, fungi, and bacteria in the rhizosphere (Fig. 1).

## Defining Mycorrhizas

Mycorrhizas are complex interactions comprising fungi belonging to different taxonomic groups and about 90% of land plants (Smith and Read, 1997). Mycorrhizas are successful in colonizing diverse environments, and their ecological success reflects a high degree of diversity in the genetic and physiological abilities of the fungal endophytes. About 6000 species in the Zygo-, Asco-, and Basidiomycotina have been recorded as mycorrhizal, but the advent of molecular techniques is increasing the number, since new mycorrhizal species as well as new associations are described on the basis of their DNA fingerprinting (Bidartondo *et al.*, 2002). The taxonomic position of plant and fungal partners defines the types of mycorrhiza, the main distinction of which is between endomycorrhiza and ectomycorrhiza. Generally speaking, the fungal hyphae in endomycorrhiza penetrate the root cells to establish an intracellular symbiosis, whereas in ectomycorrhiza the hyphae remain extracellular. However, various patterns of colonization are adopted by mycorrhizal fungi during their interactions with the host, mostly among endomycorrhizal fungi. In addition to arbuscular mycorrhizal (AM) fungi, which will be discussed in greater detail, ericoid mycorrhizal fungi colonize the root cells of their host, producing an infection unit that involves a single host cell without spreading to the neighboring root cells. In orchid mycorrhizas, intracellular coils in cortical cells are usually produced by Basidiomycetes during both the protocorm and root colonization; in this case, the infection unit comprises a larger number of host cells. Ectomycorrhizal fungi have a more recent evolutionary history; they do not penetrate the host cell wall, and they complete their colonization through two major

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events: the production of a tissue-like structure (the mantle) covering the root surface, and the development of a labyrinthine, extracellular hyphal network within the root tissues, termed the Hartig net (Bonfante, 2001).

This report focuses on arbuscular mycorrhizal (AM) fungi, which have been recently classified in a new taxon, the *Glomeromycota* (Schüssler *et al.*, 2001).

Fossil and molecular data suggest that roots and AM fungi have shared a cooperative life since Devonian times (Simon *et al.*, 1993). The success of mycorrhizas in evolution is mainly due to the central role that AM fungi play in the capture of nutrients from the soil in almost all ecosystems (Smith and Read, 1997), and in phosphate uptake in particular (Smith and Barker, 2002, for a review). As a consequence, they are crucial determinants of plant biodiversity, ecosystem variability, and plant community productivity (van der Heijden *et al.*, 1998). AM fungi are not only an essential feature of the biology and ecology of most terrestrial plants, they also interact with different classes of bacteria during their life cycles. In fact, AM fungi establish interactions both with bacteria living in the rhizosphere (Fig. 1) during their extraradical phase and with endosymbiotic bacteria that live in the cytoplasm of some fungal isolates (Perotto and Bonfante, 1997; Bonfante *et al.*, 2001).

To understand these multiple interactions and to apply

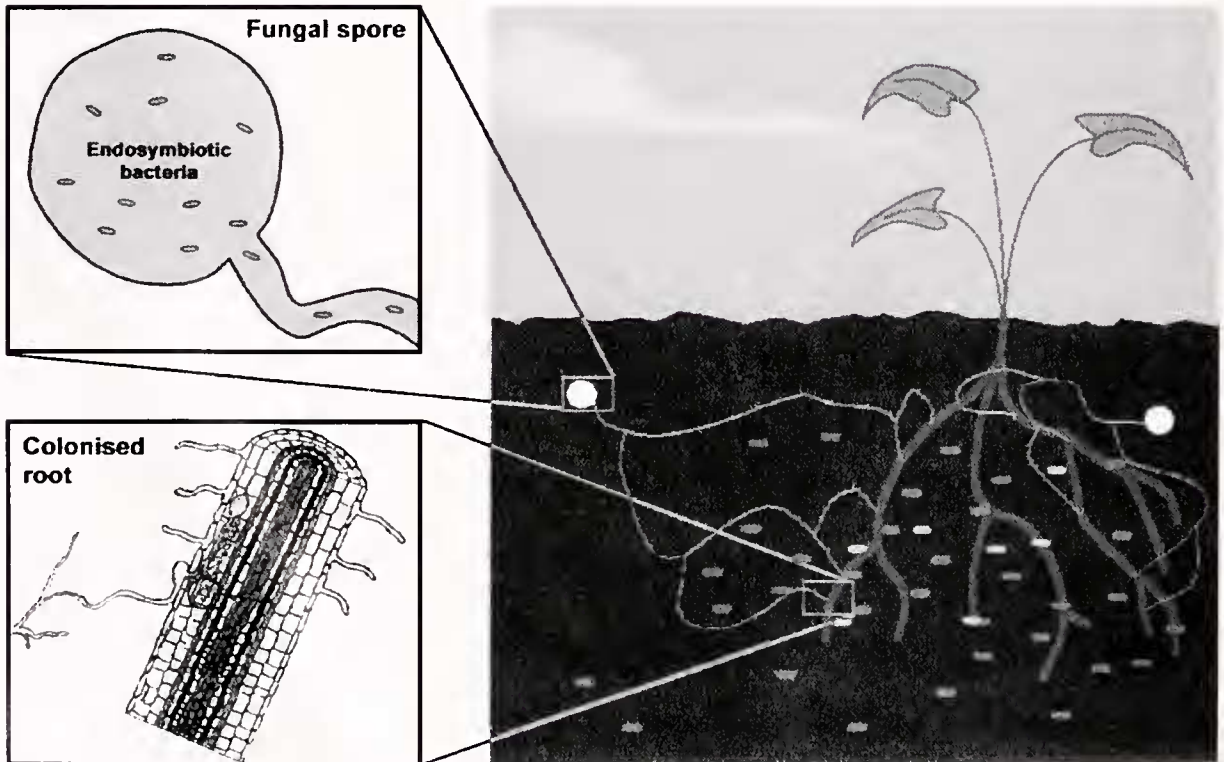
them in low-chemical-input agricultural systems is one of the most exciting challenges of current research in the field of molecular microbe-plant interactions.

### Plant-Fungal Interactions: Cells, Genes, and Signals

The impressive diversity of the plant and fungal taxa involved in mycorrhizal symbiosis has resulted in their anatomical description in many hosts since the early twentieth century (Smith and Smith, 1997, for a review). The characterization of mycorrhizal phenotypes has led to a well-defined picture of the colonization by AM fungi, the main aspects of which are summarized on the web site (<http://www.bioveg.unito.it/lotus.htm>).

The availability of plant mutants with impaired symbiotic capabilities has demonstrated that colonization is a multi-step, genetically regulated process that is under the control of specific loci (Bonfante *et al.*, 2000; Novero *et al.*, 2002). As a consequence of this process, the cellular organization of the two eukaryotes is completely remodeled. A detailed analysis of cell-to-cell interactions between host and mycobiont identifies the cell walls, membranes, and cytoskeleton of both partners as the structures where crucial changes occur (Bonfante, 2001).

However, analyzing the molecular bases of the dialogue



**Figure 1.** The scheme illustrates the multiple cellular interactions established among AM fungi, root cells, and bacteria in the rhizosphere. The magnification of the spore (insert at top left) shows the endobacteria living in the cytoplasm.

between the two partners is not an easy task. Other symbiotic systems are more advanced; for example, the interaction between *Rhizobium* and legumes is finely regulated by signal molecules, which are perceived by receptors that activate a signal transduction cascade, eventually leading to the activation of target genes and to the production of a nodule (Long, 1996). In AMs, knowledge of these steps is in its infancy; potential signal molecules have been found but not characterized (Buee *et al.*, 2000). Attention is therefore mostly focused on the genes that code for proteins responsible for the functional traits in AM fungi; for example, the phosphate transporter (Harrison and van Buuren, 1995) or a metallothionein gene (Lanfranco *et al.*, 2002). Further challenges are posed by other, obscure aspects of AM: their obligate biotrophic status, their multinuclear condition, and an unexpected level of genetic variability (Hijiri *et al.*, 1999; Lanfranco *et al.*, 1999; Kuhn *et al.*, 2001). The genome of AM fungi is in fact huge, ranging from 0.3 pg to 1.12 pg/DNA for the nucleus, depending on the species (Gianinazzi-Pearson *et al.*, 2001). For all these reasons, genomic projects have not been attempted.

On the plant side, other problems are encountered. *Arabidopsis thaliana*, the first plant genome to be sequenced, does not establish any symbiotic interactions (the *Arabidopsis* Genome Initiative, 2000). However, rice does establish mycorrhizal associations, and genomic data accrued for rice will provide significant information (Goodman *et al.*, 2002; Sasaki *et al.*, 2002). Other plants are also being investigated. Breakthroughs from recent research on genomics, involving plants such as *Medicago truncatula* and *Lotus japonicus*, have led to the availability of about 100,000 expressed sequence tags (ESTs). Moreover, the availability of mutants that are impaired in their symbiotic properties has recently led to the discovery of plant genes that code for proteins that are essential to the dialogue between plants and both symbiotic microbes—AM fungi and rhizobia (Stracke *et al.*, 2002; Endre *et al.*, 2002). The genes NORK and SYMRK belong to a large class of plant and animal genes that code for receptor complexes (Kistner and Parniske, 2002). Both SYMRK and NORK present a repeated leucine-rich motif (RLM) in their extracellular domain and an intracellular receptor like-kinase domain (RLK). The discovery of these genes—which encode plant receptor kinases required for fungal and bacterial symbioses—opens the way for detailed analysis of signal perception and downstream signaling pathways that are associated with microbial recognition (Spaink, 2002; Kistner and Parniske, 2002).

### Plant-Fungal-Bacterial Interactions

In addition to the well-known interactions between plants and fungi, mycorrhizal roots offer excellent ecological niches for other microbes; some rhizosphere bacteria adhere tightly to fungal hyphae, whereas others are directly asso-

ciated with the root surfaces (Bianciotto *et al.*, 2001; Bianciotto and Bonfante, 2002). In addition, mycorrhizal fungi may host bacteria that complete their life cycles within fungal cells. As opposed to many other eukaryotic cells, which show some level of integration with bacteria and are increasingly appreciated by ecologists and evolutionary biologists for their huge diversity (Moran and Wernergreen, 2000), fungi offer only a limited number of examples. One of the best known is *Geosiphon pyriforme*, a zygomycete closely related to Glomales. It can host cyanobacteria inside characteristic bladders in the apical hyphal region (Schüssler and Kluge, 2001).

AM fungi are unique in hosting bacteria in their cytoplasm. Intracellular structures very similar to bacteria and bacteria-like organisms (BLOs) were first described in the 1970s (Scannerini and Bonfante, 1991 for a review). Ultrastructural observations clearly revealed their presence in many field-collected fungal isolates. Further investigation of these BLOs, including the demonstration of their prokaryotic nature, was long hampered because they could not be cultured. Only a combination of morphological observations (electron and confocal microscopy) and molecular analyses allowed us to identify BLOs as true bacteria and to start unraveling their symbiotic relationship with AM fungi (Bianciotto *et al.*, 1996).

Isolate BEG 34 of the fungus *Gigaspora margarita* contains a large number of BLOs that can be easily detected by staining with fluorescent dyes that are specific for bacteria and can distinguish between live and dead ones. On the basis of the 16S rDNA sequences, the bacterial endosymbionts living in *G. margarita* (BEG 34) were first identified as belonging to the genus *Burkholderia* (Bianciotto *et al.*, 1996). As a further step, on the basis of the 16S rDNA amplified from isolates of *Scutellospora persica*, *S. castanea*, and *G. margarita*, a strongly supported clade was obtained, which contained all endosymbiotic bacteria so far sequenced in Gigasporaceae. It was located close to the genus *Burkholderia*, as well as to the genera *Ralstonia* and *Pandorea*. A new bacterial taxon was therefore proposed: *Candidatus Glomeribacter gigasporarum* (Bianciotto *et al.*, 2003). The results demonstrate that endobacteria are widespread in Gigasporaceae, and suggest that they represent a stable cytoplasmic component. Preliminary results showing that bacteria move along with the fungi from one generation to the next, following a vertical transmission mechanism (V. Bianciotto and G. Bécard, unpubl.), provide a first experimental confirmation of the statement. A number of morphological observations showing bacteria living inside *Glomus* spores and hyphae (Scannerini and Bonfante, 1991) might suggest that endobacteria are not limited to the Gigasporaceae. However, attempts to obtain ribosomal sequences and to identify these endophytes on the basis of their DNA sequences have been so far unsuccessful, suggesting that

these bacteria are limited in number or, if present, belong to a mixed population.

The functional significance of AM fungal endobacteria is not clear; many attempts to cultivate them have been unsuccessful. The finding that a genomic library developed from *G. margarita* spores also has bacterial sequences (van Buuren *et al.*, 1999) helped us to identify some genes belonging to *Candidatus Glomeribacter gigasporarum*. Among the bacterial genes so far identified, the most interesting are those involved in nutrient uptake (*i.e.* a putative phosphate transporter operon, *pst*); in colonization events by bacterial cells (*vac*); and in chemotaxis (Ruiz-Lozano and Bonfante, 1999, 2000; Minerdi *et al.*, 2002). A DNA region containing putative nitrogenase coding genes (*nif* operon) was also found (Minerdi *et al.*, 2001), but these genes have not yet been demonstrated to belong to the *Candidatus Glomeribacter* genome.

### Conclusions

In conclusion, the analysis of the multiple interactions established by AM fungi with plant and bacterial cells offers new keys for understanding the complexity of AM symbiosis. In addition to the still-open question about the signal molecules produced by AM fungi and recognized by potential receptors, the widespread presence of bacteria inside or specifically associated with AM fungi suggests that many AM symbioses are tripartite associations. This possibility will lead to the definition of new parameters in the design of mixed inocula, while the identification of fungal strains that contain endosymbiotic bacteria with important genetic traits opens up new strategies for the practical use of AM fungi.

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## Discussion

QUESTION: Can you cure the fungal symbiont of the bacterial symbiont and then look at the differential effect of the fungus with and without the bacterial symbiont or the plant?

BONFANTE: This is a crucial question. We are facing the problem together with Dr. G. Becard from Toulouse. Dr. Becard's group has demonstrated that the bacteria are not sensitive to many antibiotic treatments. In fact, some antibiotics seemed to increase bacterial growth.

QUESTION: Can you grow the fungus in nitrogen-free medium?

BONFANTE: Yes, arbuscular mycorrhizas fungal spores usually germinate in water. It is well known that the first growth steps (non-symbiotic phases) proceed without other nutrients.

COMMENT (JOHN HOBBIIE): In Europe, scientists seem to appreciate—much more than in the U.S.—the importance of the mycorrhizal fungi in incorporating nutrients into shrubs and trees. A recent book points out that, in forests, most of the nitrogen and phosphorus enters the trees by way of the mycorrhizal fungi. It is also well known that most of the forests of the world are nitrogen limited. Most research until now has involved the uptake of phosphorus by mycorrhizae. Plants grown with mycorrhizae clearly grow much better than those grown without, but in many cases nitrogen could have had an effect as well. What the fungi can do,

of course, is to get at the organically bound nitrogen, which is most of the nitrogen in the soil. This organically bound nitrogen is not available to the plant without the microbial “mineralization” to ammonium and nitrate, or without an enzymatic breakdown to amino acids, uptake, and transport to the roots by the mycorrhizal fungi. The fungi obtain sugars from the tree roots and provide nitrogen, phosphorus, and even water to the tree or shrub. Up to 30% of the carbohydrates fixed in photosynthesis can be transported under ground and respired by the fungi.

The importance of the symbiosis between plant roots and mycorrhizal fungi is just beginning to be investigated. Ecologists want to know about the regulation of the fungal breakdown of proteins and other nitrogen compounds, and how much of the total pool of organic nitrogen in soil is available.

BONFANTE: While the role of AM fungi in phosphate uptake is largely acknowledged, their role in nitrogen uptake is a recent discovery. Fitter's laboratory recently published an interesting article in *Nature*, which shows that AM fungi use an organic N source<sup>1</sup>. The role of ectomycorrhizal fungi in nutrient cycles is

<sup>1</sup> Hodge, A., C. D. Campbell, and A. H. Fitter. 2001. An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. *Nature* **413**: 297–299.

well demonstrated, and molecular mechanisms are being explored. The most recent views on these topics can be found in the book by van der Heijden and Sanders<sup>2</sup>.

QUESTION: Is there any evidence yet that, in a forced succession, there might be a concomitant succession of fungi in the soil?

BONFANTE: Under natural conditions, a fungal succession is

known to accompany that in the roots. This has been well described in ectomycorrhizae: early colonizers and later colonizers have been identified in many plant communities. The development of molecular probes is now providing new views and opened new questions on the identification of spatial and temporal factors underlying community structures<sup>3</sup>. The new approaches of molecular ecology will allow us to directly monitor the fungal succession under microcosmal conditions.

<sup>2</sup> van der Heijden, M. G. A., and I. R. Sanders, eds. 2002. *Mycorrhizal Ecology*. Ecological Studies, Vol. 157. Springer, New York.

<sup>3</sup> Dahlberg, A. 2001. Community ecology of ectomycorrhizal fungi: an advancing interdisciplinary field. *New Phytol.* 150: 555.